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Review

Updating traditional regulatory tests for use with novel materials: Nanomaterial toxicity testing with *Daphnia magna*

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A B S T R A C T

Nanomaterials (NMs) are being widely incorporated into a variety of fields such as medicine, cosmetics and electronics, due to the exceptional qualities provided by their small size and high surface area to volume ratio. Increased use of NMs leads to their deposition into environmental waters where they interact with organisms such as the fresh water zooplankton *Daphnia magna* (*D. magna*). *D. magna* is an ideal candidate for fresh water toxicity testing and a central study species used by the Organization for Economic Cooperation and Development (OECD) which sets the gold standard for regulatory testing protocols. The ecotoxicity protocols using *D. magna* were originally designed for bulk chemicals though have been deemed acceptable for NM testing, despite NMs existing as suspensions rather than dissolved chemicals. These protocols fail to account for key exposure features of NMs such as the fact that the natural clearance processes in these organisms require food to push out previously accumulated matter, and that under realistic exposure scenarios, NMs will have acquired a biomolecule corona that changes their identity, stability, uptake and excretion. Thus, the lack of biomolecules added to the medium and lack of feeding can lead to significant over or underestimation of the amount of NMs taken up by, or retained within, *D. magna* leading to uncertainty of dose and ultimately miscalculation of NMs toxicity and the risks posed by these materials. Herein we present evidence to support the call for revised guidelines for *D. Magna* acute and chronic toxicity tests for hazard and risk assessment of NMs.

1. Introduction

Nanomaterials (NMs) are widely incorporated into a vast range of consumer products already on the market such as zinc oxide (ZnO) NMs incorporated into sunscreen due to their Ultra-violet deflection properties (Kokura et al., 2010), cerium dioxide (CeO_2) NMs in catalytic converters in cars (Montini et al., 2016), titanium dioxide (TiO_2) NMs as a food colouring agent (Periasamy et al., 2015) and gold (Au) NMs for cancer therapy due to their high photo-thermal capacity (Kennedy et al., 2011). NMs are also being incorporated into several emerging potential applications such as carbon nanotubes (CNT) for drug delivery due to their hollow interior for drug loading and ease of passing through blood vessels (Liu et al., 2009). As such, NMs are increasingly likely to be deposited into environmental waters and therefore have an increased chance to interact with freshwater species. Indeed, predicted environmental concentrations (PEC) of NMs in surface waters are in the ng/L range, specifically for plastic based NMs such as polystyrene whose PEC ranges from 0.001 to 0.8 ng/L (Mueller and Nowack, 2008).

The novel characteristics exhibited by NMs, due to their small size giving them a high surface area to volume ratio, that make them ideal candidates for use in industrial processes such as catalysis and sensing, may be the same characteristics that impose a toxic effect towards biological organisms by increasing their “accessibility” to critical biological functions and their reactivity. Additionally, toxicity of NMs is dependent on several factors such as the core composition, the surface coating, charge and shape such that toxicity (both at a cellular and organism level) of one form of a NM may not be predictive of another form of NM of similar core composition (Nasser et al., 2016, Zheng et al., 2017, Zhang et al., 2016). This is further confounded by the dependence of toxicity on cell type or organism being exposed, as well as the exposure conditions (ionic strength, pH, presence or absence of natural organic matter etc. (Lynch et al., 2014)). This duality has been described as NMs possessing both intrinsic and extrinsic modes of toxicity. Intrinsic toxicity of NMs is related to the properties of the NM itself such as size and shape, while extrinsic toxicity is the behaviour of the NM and its interactions with surroundings such as agglomeration in exposure medium (including constituents within this), causing secreted biomolecules present in the exposure medium to affect the extrinsic toxicity of the NMs. Of course, the extrinsic toxicity influenced by the exposure medium is facilitated by the intrinsic properties of NMs. For example, shape is an intrinsic property where it has been shown that Au rod-shaped NMs are more toxic compared to Auspheres at the same number concentration (Nasser et al., 2016), although differently shaped gold NMs have different stability in medium and therefore may agglomerate differentially (Wang et al., 2014), altering their uptake and toxicity towards *D. magna* so that the intrinsic features of the NM govern the extrinsic. The fact that so many NMs physico-chemical and environmental factors can contribute to the mode of action or toxicity

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means that a range of parameters need to be varied systematically and assessed in order to identify the specific driver(s) of toxicity.

Due to the increasing production and use of NMs it is imperative to have precise operating procedures in order to assess and compare toxicity from different chemicals or NMs in terms of their impacts on agreed end-points. These end-points and standardised assays should then provide a clear representation of how any given NM would interact with the selected environmental organisms to accurately determine the hazard posed by the NM and from there to determine the overall risk (Hazard × Exposure). This has long been the goal of the Organization for Economic Cooperation and Development (OECD), and indeed is successfully implemented for chemicals. However, despite a decade of research into the applicability of OECD assays for NMs (Hjorth et al., 2017, Hansen et al., 2017), including the global OCED sponsorship programme which produced dossiers on the 12 NMs identified as having the highest industrial relevance due to their production levels (Hansen et al., 2017), it is only now that real progress is being made towards updating regulatory guidance (e.g. the ECHA recommendations in 2017 and the subsequent drive to have the OECD test guidelines amended for NMs by 2020 to allow implementation of the nano-specific amendments to the RECH guidelines). To support this process, we present here a summary of 3 years of research into the applicability of the existing regulatory protocols for NMs, and emerging or future NMs, focussing on the acute immobility test with *D. magna*.

2. Regulatory test guidelines for *D. magna* and their suitability for NMs

D. magna is a fresh water filter-feeding organism that takes up particulates that are present in the water column and thus are exposed to a wide range of matter such as released NMs; as such they are at the heart of eco-toxicity test guidelines. *D. magna* is an indicator species as they are sensitive to environmental pollutants which cause changes to the organism at both internal cellular levels (Wang et al., 2009) and at the physical level affecting shedding of their external carapace (Zou and Fingerman, 1997, Ma and Lin, 2013), inducing alterations to swimming behaviour (Noss et al., 2013) or potentially impacting on their feeding ability. *D. magna* are also a keystone species and have an important place in the food chain, acting as a food source to a number of organisms as well as consuming algae and controlling algal bloom levels. From a toxicological model viewpoint, the most ideal characteristic *D. magna* possess is their asexual (parthenogenetic) reproduction which causes offspring to be genetically identical, ultimately decreasing variation in experimental results, along with being transparent which makes it easy to observe internal changes under a microscope. *D. magna* have been used as a model organism long before NMs emerged as an environmental pollution issue, which is the crux and focus of this review; while *D. magna* is an ideal species to be used for chemical toxicity testing, it is not clear that the current protocols are suitable for NMs and thus we suggest how they can be adapted to correctly represent NMs for robust risk assessment.

The OECD is the gold standard for fresh water toxicity testing of chemicals towards *D. magna.* Two of the commonly and widely used protocols for *D. magna* toxicity testing are the short term acute immobilization test (OECD 202) which looks at death in the form of immobilization between 24 and 48 h, and the longer term chronic reproduction test (OECD 211) looking at reproduction over 21 days. These protocols provide stringent instruction for what is appropriate for exposure conditions, giving specifics on test solutions, conditions of exposure and exposure duration. An important factor that this review investigates, is how the lack of food in the OECD 202 guideline can over or underestimate the residency time of NMs, and therefore toxicity of NMs, causing a miscalculation of risk. Currently, the OECD 202 protocol only requires details of the food source to be stated during the culturing period of *D. magna* as part of describing the test species parameters, with no mention of food during the test conditions (OECD, 2004). While *D. magna* use peristaltic contractions to move food and other ingested material along their gut, the main source of material movement through the gut passage is the pressure provided by newly ingested material. The lack of food present in the 24 h following (or during) NM exposure during the OECD 202 acute test potentially inhibits complete depuration and can thus result in higher levels of NMs being retained within the gut causing an overestimation of the residency time and toxicity of NMs. The OECD 202 study was last updated in 2004, over a decade ago and notably, before the first significant reports of potential ecotoxicity from NMs emerged (Colvin, 2003). Thus, it is timely to collect current knowledge and develop an updated protocol that takes account of the unique features of NMs and their high surface reactivity and interaction with biomolecules. Consideration of factors such as NMs agglomeration, and binding of natural organic matter and secreted biomolecules, would allow for environmentally realistic exposure conditions to be used, and would facilitate inter-laboratory comparison and comparability of the toxicity of different NMs based on their EC_{50} values for example.

While the sentiment of fluidity between protocols inherent in the OECD approach is current and appropriate for bulk chemicals as originally designed for, these same protocols may not be applicable for NMs and are thus under re-evaluation to assess their suitability and applicability for NMs. Importantly, NMs have been agreed to be chemicals, and as such there is no legal requirement for separate regulation, but it is acknowledged that due to their high surface area and reactivity the standard tests may need adjustment. The focal issue is that aquatic ecotoxicity testing with NMs involves exposure of *D. magna* to NMs which are present as a suspension, rather than dissolved into solution as is the case for molecular chemicals for which the OECD guidelines were originally developed (Petersen et al., 2015). These suspended NMs are subject to interaction with their surroundings and undergo transformations such as agglomeration and dissolution which are contingent on the type of NM and the surrounding medium (Handy et al., 2012). NMs that dissolve rapidly are most likely suitable for standard equilibrium tests though this is entirely dependent on where they dissolve. NMs existing within media which are then taken up by *D. magna* will be exposed to a decrease of pH within the organisms gut which may cause dissolution to occur post-uptake, causing an increased exposure and toxicity (the so-called Trojan horse effect), compared to if *D. magna* is exposed to the ionic form directly in the medium. This enhanced uptake arises because *D. magna* are more likely to take up entities (such as NMs, especially agglomerated NMs closer in size to their natural food source) than ions which once consumed, may dissolve within the gut, leading to increased toxicity (Briffa et al., 2018). These NM-specific factors can result in large inconsistencies in exposure-response approximation and an inaccuracy in determining the potential risk of NMs, if not considered and controlled. For instance, agglomeration of NMs can alter their settling rates and thus availability for uptake (Petersen et al., 2015), confounding dose-response relationships based on the exposure mass without consideration of the proportion of dispersed versus agglomerated NMs and the settling rates.

In order to assess the suitability of NM testing using OECD guidelines, a working party on manufactured nanomaterials (WPNMs) was established in 2007 by the OECD to solidify the opinion that the current protocols were appropriate for NM testing. The WPNM deliberated that the protocols were fitting for NMs though certain measures needed to be incorporated to adapt them for NMs; however these measures were never clearly defined and only minimal tangible modifications were made with regards to the overall sample preparation within the test guidelines general considerations. Indeed as noted above, there has been no update to the *D. magna* test guidelines since 2004.

A limited number of groups who work with NMs have documented alterations needed to the OECD guidelines. For example, sedimentation of NMs in the short-term OECD 202 acute toxicity tests may result in reduced exposure of *D. magna* which can be ameliorated by testing at pHs which show stable dispersions and in a low ionic-strength medium

(Hund-Rinke et al., 2016). One group suggested that immobilization as an end point may not be appropriate as binding of NMs to the *D. magna* carapace may render the organism immobile without being dead, leading to a false-positive result of mortality (Rosenkranz et al., 2009). Since *D. magna* use their appendages to create a current of water over their respiratory surface, binding of NMs to these appendages may in turn suffocate the organism rendering it immobile even before ingestion (Handy et al., 2012). NMs such as TiO_2 and Au have both been shown to prevent *D. magna* from shedding their carapace (Dabrunz et al., 2011, Nasser et al., 2016), another form of physical toxicity potentially independent of ingestion, or indicative of diverted energy due to toxicity.

Currently, the OECD 202 test does not take into account that natural waters contain biomolecules which can bind to NM surfaces creating an eco-corona which can cause stabilization/destabilization of NMs dispersions. Even in biomolecule-free medium, *D. magna* themselves secrete proteins and carbohydrates from their guts and via their moulting fluid, causing the surrounding medium to constantly be 'conditioned'. The interactions of these biomolecules with NMs can lead to stabilisation, re-dispersion or destabilization of the NMs, and means that the toxicity experiments are themselves dynamic and the NMs at 12 h, and their interactions with the organism, will be very different from those in the first couple of hours, which confounds the interpretation of the toxicity. In cases where the biomolecules causes agglomeration and an increase in NM size, the result is often that these larger NMs are a more attractively sized food source for *D. magna* than the individually dispersed NMs, thus enhancing uptake (Nasser and Lynch, 2015). NMs with an acquired corona may get taken up to a higher degree than pristine NMs (due to the above-mentioned agglomeration), but also have an enhanced retention within the gut of *D. magna* (Nasser and Lynch, 2015) making *D. magna* feel full for longer, potentially leading them to delayed development and, if sustained, to starvation. Ultimately, the OECD standard protocols may not be adequate for use with NMs on several grounds, as depicted in Fig. 1, as they were originally designed for solutions and are based on chemical potential driven equilibrium distributions and not for NM suspensions which have a high surface area that bind to biomolecules, are potentially taken up through active processes rather than simply diffusing into the gut, and indeed may be further internalised by receptor mediated process from the gut.

The focus of our analysis of the impacts of NM-specific factors on the applicability of the OECD test guidelines for NMs, includes: (1) the lack of feeding at different time-points during the feeding cycle of *D. magna* which may lead to an over or underestimation of the residency time of NMs within the gut and to altered toxicities; and (2) the lack of natural biomolecules in the standard test media, despite the ubiquity of biomolecules in environmental waters which inevitably bind to the NMs causing changes to the NM surface and creating an 'eco-corona' leading to altered stability/instability of NMs and ultimately affecting NM uptake and toxicity. These factors are further discussed below.

3. Impact of food on NM uptake and depuration

A significant amount of research has been conducted investigating the effects of NM uptake and toxicity towards *D. magna* under OECD guidelines; some examples are detailed in Table 1. It is clear from Table 1 that none of these studies consider the need for a food source to be present, which influences both the uptake/depuration and toxicity. Based on our findings, whereby the depuration of 500 nm polystyrene NMs occurs up to 1.7 times faster in the presence of a food source during the excretion phase compared to exposure without any food source present in the first hour post-exposure (Nasser et al., unpublished data), it is clear that the presence of food needs to be considered when adapting traditional testing methods created for bulk/soluble materials for use with NMs. The lower retention of the particles suggests enhanced removal in the presence of food (Nasser et al., 2019).

The presence of a food source at different time points during the feeding and excretion cycle for *D. magna* can drastically influence the amount of NMs taken up and retained within *D. magna.* The presence of a food source can influence NM uptake and excretion in three scenarios; (1) presence of food alongside NM exposure; (2) food applied after NM consumption; (3) presence food within the gut of *D. magna* before exposure to NMs, all of which are detailed below. Ultimately the accumulation of NMs within an organism will be a function of environmental conditions, organism properties (such as being a filter feeder) and the intrinsic NM properties (Wray and Klaine, 2015).

The longer chronic toxicity tests (OECD 211), lasting 21 days, do include feeding at least once a day (although some studies report feeding every 3 days), although this step is included with the intention to keep the *D. magna* healthy and not with the purpose to influence NM uptake and excretion. Shorter acute toxicity tests (202) as previously mentioned, have a complete absence of food which disregards the mechanism of clearance in these organisms, which requires the presence of food to provide the pressure that pushes out previously ingested food. Thus, we suggest that in the absence of food (algae) a small amount of NMs will get trapped within the gaps of the bush boarder, as shown schematically in Fig. 2, while co-exposure with algae will reduce this as the larger algae cannot enter these gaps. This absence of food can thus lead to retention of NMs within the gut at the end of experiment, and does not mimic realistic environmental waters where food sources are abundant.

It is also worth noting that many studies do not indicate when the last feeding prior to NM exposure occurred (see Table 1). If there is already food present within the gut this can cause *D. magna* to feel 'full' resulting in less NM uptake, highlighting the fact that more detailed reporting is essential to be able to compare studies and to accurately determine risk of NMs.

Several studies have shown the interaction of NMs with microvilli comprising the bush border in the absence of food as outlined in Table 2, and it must be considered that the presence of food would change the location of these NMs. It has been suggested that the elimination of NMs occurs as a two-part model whereby the majority of NMs (up to 70–90%) are eliminated rapidly, termed the 'fast compartment' (Wray and Klaine, 2015). The particulates (food or NMs) within the 'fast compartment' have an exit route through the main lumen via peristaltic contractions but more importantly are pushed out by incoming food, so that the majority of food/NMs existing within the main lumen are eliminated. Dissolved chemicals, unlike dispersions, are not retained within the gut in the same manner as they simply diffuse out with the water in a passive manner, and therefore the presence of a food source is not as vital when considering non-particulate substances. Indeed, it has also been noted that smaller non-agglomerated NMs may also passively diffuse into the gut even in the absence of active feeding.

The remaining 10–30%, termed the 'second compartment' is the much slower removal of NMs, which presumably are the NMs that are lodged within the gaps of the bush boarder (Wray and Klaine, 2015). For example, it has been shown that 500 nm Polystyrene (PS) particles reside mostly within the lumen with a minimal amount sliding into the gaps in the bush border and becoming lodged there, as seen in Fig. 3, where they are retained over longer periods. In the absence of food there is no incoming pressure other than peristalsis to potentially push out previously ingested NMs. The width of the gaps between microvilli in the bush boarder is calculated from the TEM images in Fig. 3(a) to be just below 2 μm and the depth of each microvilli is calculated to be approximately 4.6 μm from Fig. 3 (b), such that NMs, including agglomerates < 2 μm, have the potential to enter the gaps, and due to the peristalsis they get pushed further down into the gaps and get stuck there.

The position of the small fraction of NMs in the bush border make it difficult for newly incoming food to come in contact with them and therefore they are likely not pushed out of the gut in the same fashion as those existing within the main lumen via the 'fast compartment'. Indeed they may get increasingly pushed into the microvilli as a result of the

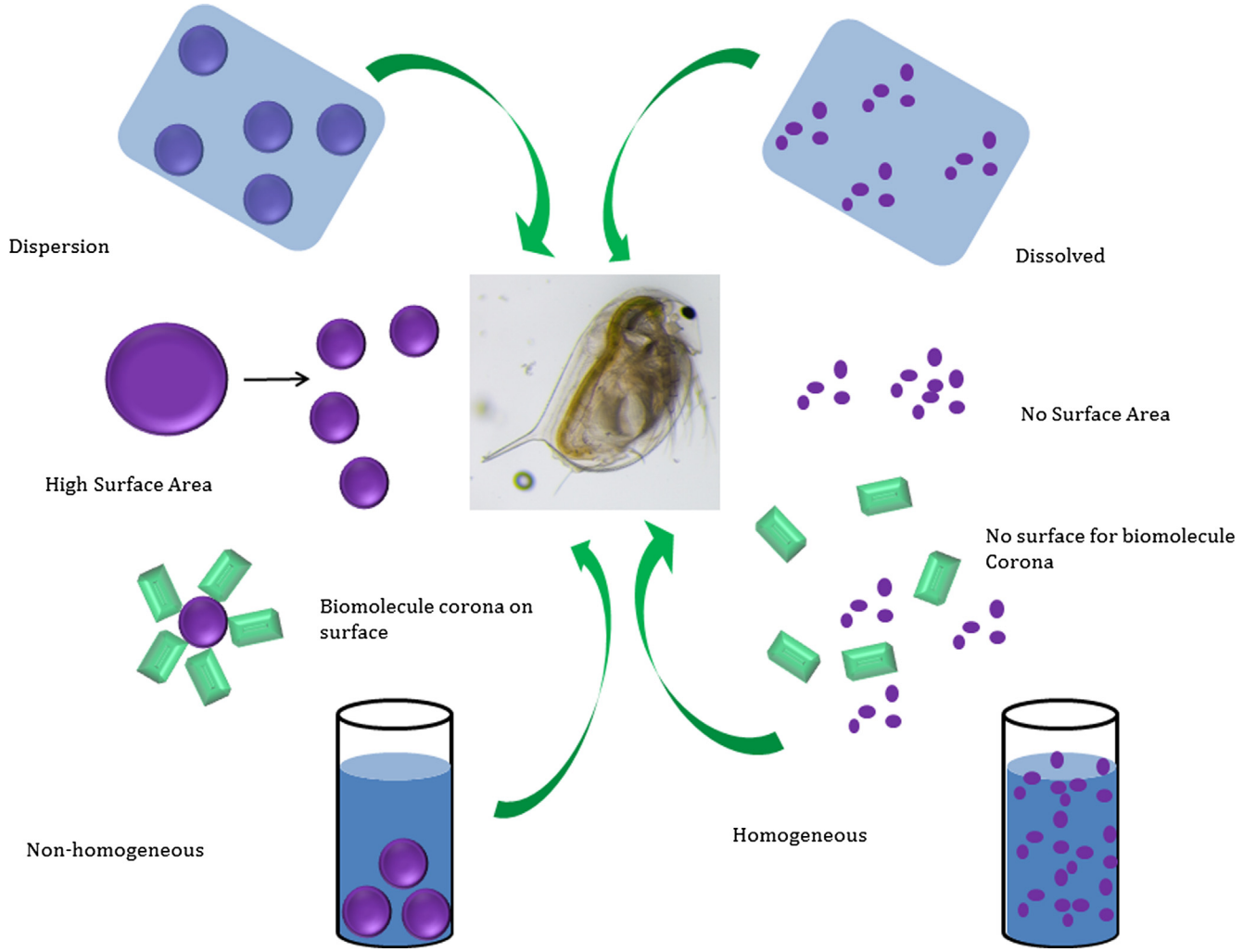

Fig. 1. Differences between NM suspensions (left of *D. magna*) and dissolved chemical solutions (right of *D. magna*) and how they interact with *D. magna*. The large surface area of NMs leads to the acquisition of a bio-molecule corona which affects both the NM properties (e.g. stability, agglomeration) and may make them a more attractive food source. Additionally, NMs are potentially taken up through active processes (driven by their acquired corona) rather than simply diffusing into the gut and indeed may be further internalised by receptor mediated process from the gut.

Table 1
Studies utilizing the short term acute OECD 202 or chronic OECD 211 test with complementary uptake studies of NMs in either the absence or presence of a food source. AAS – Atomic Adsorption Spectroscopy; ICP-MS – Inductively Coupled Plasma Mass Spectrometry; TEM – Transmission Electron Microscopy.

NM	OECD 202 (acute) or 211 (chronic) study	Complementary uptake studies related to OECD 202 (acute) test	Reference
TiO_2, uncoated, 80% anatase/20% rutile, 21 nm	OECD 202 acute, 48 h continuous exposure, daphnids 6–24 h old at start of exposure, **absence of feeding** during NM exposure. EC_{50} > 100 mg/L OECD 211 chronic, 21 days continuous exposure, *D. magna* 6–24 h old, feeding on *pseudokirchneriella subcapitata* with NMs and food added together every 3 days.	Uptake determined via time-weighted mean at 24 and 72 h, and following 6, 12, 24, 48 and 72 h depuration. **Absence of food** during both NM exposure and depuration phases. *D. magna* 8 days old initially, time-weighed.	Zhu et al. (2010)
TiO_2, uncoated, 100–200 nm	OECD 202 acute, 96 h, < 24 h at start of exposure. **Fed 90 mins prior to test though not during**. EC_{50} 0.73 mg/L	Uptake at 0, 1, 2, 3, 6, 12, 24, 48, 72, 96 h quantified by ICP-MS	Dabrunz et al. (2011)
ZnO, 99.6% purity, 20 nm	OECD 202 acute, 48 h continuous exposure, *D. magna* < 24 h, **absence of feeding** EC_{50} 0.622 mg/L	Uptake assessed after 24 and 48 h exposures, *D. magna* < 24 h old at start of exposure, light microscopy. **Absence of food and no depuration phase**	Zhu et al. (2009)
ZnO, coated, > 96% purity, < 200 nm	OECD 202 acute, 48 h continuous exposure, *D. magna* < 24 h old, **absence of feeding** EC_{50} 1 mg/L	N/A	Wiench et al. (2009)
Ag, 220 nm	OECD 202 acute, 48 h continuous exposure, *D. magna* < 24 h old, **absence of feeding.** EC50 4.67 μg/L OECD 211 chronic, 21 days, feeding on algae *C. reinhardtii* every 3 days	48 h uptake. **Absence of food**. Uptake quantified by AAS. *D. magna* < 7 days hours old at start of exposure	Zhao and Wang (2011)
CuO	OECD 202 acute, 48 h continuous exposure, *D. magna* newly hatched, **fed on alga Spiruilna 2 h prior to testing and none present during exposure**	Uptake assessed at 10 min, 2, 18, 24, 36, and 48 h. Daphnids were **fed on alga Spiruilna 2 h prior to exposure**. TEM epoxy-resin imaging. *D. magna* newly hatched at start of exposure	Heinlaan et al. (2011)
Bimetallic Ag-Au, 41 nm, 20:80 metal ratio	OECD 202 acute, 48 h, *D. magna,* **absence of feeding** EC_{50} 70 mg/L	N/A	Li et al. (2010)

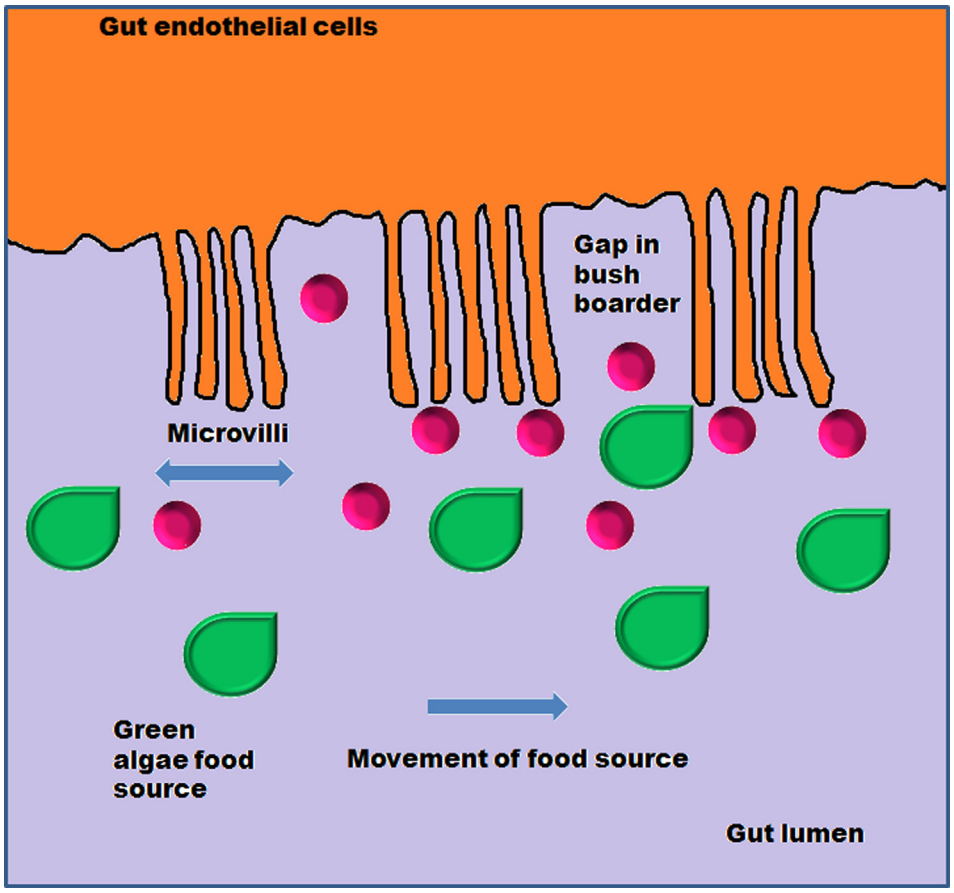

Fig. 2. Peristaltic contractions of microvilli within the gut moves NMs (pink spheres) that have been taken up through the gut lumen, although the NMs are small enough that a portion may enter the gaps in the bush border and get stuck (see also Fig. 3 for electron microscopy evidence of particles in the bush border). Incoming food (green shapes representing algae) push the NMs through the gut lumen and reduce the amount that enter the gaps in the bush border, although a minimal (but measureable) amount of NMs still get trapped. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

peristaltic motion. Interestingly, the absence of food during the initial exposure of NMs to *D. magna* allows for these NMs to become trapped within the 'second-compartment' and may over-estimate the ultimate body-burden of NMs due to the lack of food guiding them along the lumen, which may lead to an unrealistic and extended residency period of NMs within *D. magna.* Unlike soluble chemicals for which the protocol was originally intended, the high surface energy of NM surfaces cause them to a have a high affinity to biological surfaces such as algae even before exposure to *D. magna* as well as giving them a degree of stickiness once within the gut to bind to the gut wall and gaps of the bush boarder rendering them difficult to remove.

In reality, NMs will be taken up incidentally along with food, which allows them to pass through the gut lumen more efficiently as the NMs are intertwined within the algae and move through the gut lumen along with the food. The likelihood of NMs being deposited into environmental waters where traditional food sources for *D. magna* such as the algae *Cholera vulgaris* (*C. vulgaris*) co-exist and are incidentally taken up along with the food source is extremely high. This has been seen in several cases where Ag NMs were taken up into the gut of polychaetes when exposed to NMs via their food (García-Alonso et al., 2011) and was also seen with Ag NMs and gastropods (Kalman et al., 2015). Without a food source present during the exposure phase, NMs may be taken up less or in a passive fashion, underestimating rates of uptake that would actually occur in a natural environment. The presence of food such as *C. vulgaris* acts as a contamination sink for NMs which may adsorb to algal surfaces and therefore the presence of a food source may facilitate the unintentional uptake of NMs by *D. magna* when they consume algae (Kalman et al., 2015). In parallel, NMs that enter bound to a food source remain intertwined due to their high affinity towards organic matter, such as algae and pass through the gut and are finally excreted with the food source. It has been shown that *D. magna* has a higher rate of egestion of Ag NMs presented with food creating faecal production compared to the same NMs presented only with water (Zhao and Wang, 2010). Similarly, Ag NMs which were associated with algae during the exposure phase had a dietary assimilation (the transfer of nutrients into an organism after digestion in the gut) of 22–45% which was much higher than the removal of Ag NMs presented without algae (Zhao and Wang, 2010).

Collectively, the evidence presented here indicates the need to include a food source in the experimental design in order to obtain realistic values of NM bioaccumulation (when an organism takes up/absorbs substances faster than they excrete) within the gut and how this relates to toxicity as pertaining to the original OECD guidelines' aim.

The addition of a food source needs to be considered in order to improve current protocols whilst not jeopardising other areas of the protocol. The amount of food being provided needs to be appropriate for the amount and size of *D. magna* exposed so as not to cause over feeding. The presence of an ideal food source in large quantities can

Table 2
Studies showing interaction of NMs with microvilli comprising the bush boarder under default OECD (absence of food) conditions.

Conditions showing NMs adhering to microvilli within *D. magna* bush boarder	Reference
6 and 20 citrate coated Au spherical NMs, **absence of feeding**	Wray and Klaine (2015)
30 nm CuO NMs, **absence of feeding**	Heinlaan et al. (2011)
50 and 500 nm PS NMs comparing **absence or presence of feeding**	Nasser and Lynch, upublished data

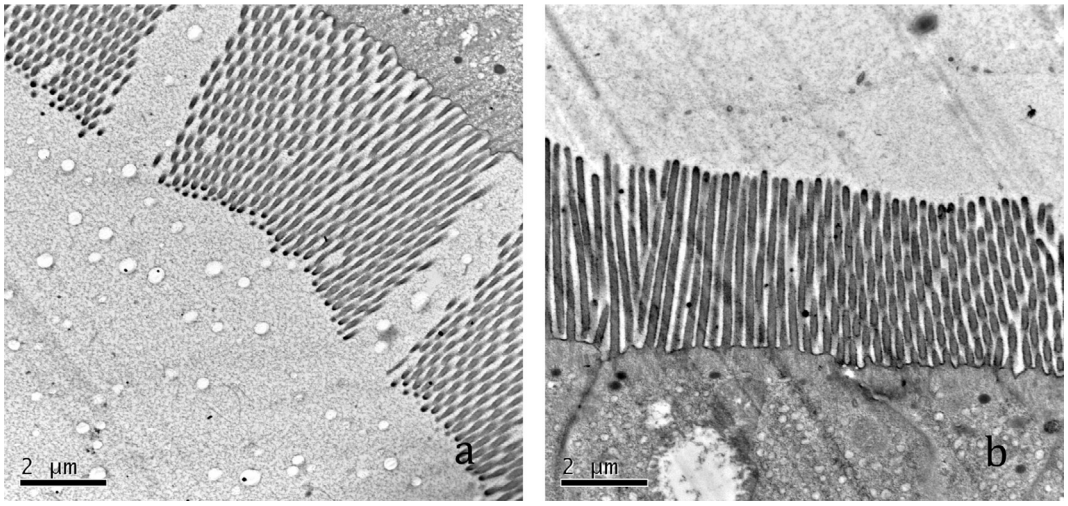

Fig. 3. 500 nm PS particles taken up into the gut lumen of *D. magna* without any food source: the majority of the NMs exist within the gut lumen and a minimal amount enter and get stuck in the gaps of the bush border.

lead to overfeeding resulting in full gut leading to a decrease in subsequent feeding of algae that may be intertwined with NMs providing an underestimation of NM uptake and therefore the presence of a food source should only be to facilitate realistic conditions where there would be strong competition for food. Herbivores such as *D. magna* face nutritional challenges because plant matter relative to animal tissue is relatively low in nutritional content, usually assessed in calories/gram as well as elemental amounts such as the carbon (C) and nitrogen (N) ratio. Thus, the addition of food during NMs exposure should be adequate to maintain healthy broods, whilst also facilitating NM uptake and depuration. A recommendation of feeding once a day as per usual and then having a suitable dose of food present during exposure would be a beneficial alteration providing more environmentally realistic results.

4. Presence of secreted biomolecules and natural organic matter

The high surface area presented by NMs provides them with a high absorption capacity whereby naturally existing biological matter found in freshwater systems, such as natural organic matter (NOM), binds to NM surfaces in order to reduce their surface energy (Kelly et al., 2003). The notion of medium existing completely free of biomolecules is totally unrealistic, as organisms such as *D. magna* themselves secrete biomolecules such as carbohydrates and proteins during the shedding of the exoskeleton, as well as conditioning their surrounding medium by the secretion of proteins including from their gut microbiomes (Briffa et al., 2018, Nasser and Lynch, 2015)) and the release of kairomones as a natural process of predator-prey responses. Thus, utilising media free of biomolecules is unrealistic and adds to the difficulty of interpreting NM toxicity, since as noted above the NMs will change over the exposure duration. Currently, there is an OECD guideline for dispersion stability of NMs in simulated environmental media, though it is still classed under the main heading of a guideline for testing chemicals, although it does go on to accept that NM behaviour is governed by NM characteristics as well as the characteristics of the suspension medium (OECD, 2017). The new test guideline urges testing of the stability of NMs in the presence of NOM although this is a static amount pre-set before the experimental set-up and does not take into account NM concentration or size and therefore ignores surface area and binding capacity such that there may not be complete surface coverage under some circumstances. This is also unrealistic given the dynamic exchange of biomolecules actually present in 'real-life' conditions due to biomolecules being excreted as a function of time over a short period by the test organisms, the nature of which will change in response to different stresses on the organisms. Representative European freshwaters, such as Class I to VI are test media used in NM and colloidal studies (Hammes et al., 2013), whose compositions span the hardness, ionic strengths and NOM contents of waters throughout Europe, make suitable alternatives to standard OECD test media. Dissolved organic carbon (DOC) is the fraction of NOM that has the largest influence on NM stability and ranges from as low as 1.84 mg/L in class I water to as high as 12.48 mg/L in class II water (Hammes et al., 2013). However, part of the reason the OECD excludes NOM from their tests is that it is highly irreproducible and complex in composition. Thus, the compromise that we present below is utilisation of organism conditioned medium, the concentration of proteins in which can be controlled by determining the number of organisms and the volume of medium being conditioned. Previous studies using 10 *D. magna* neonates to condition 5 mL of medium reached protein concentrations of 140 µg/mL (Nasser and Lynch, 2016), considerably higher than the DOC concentrations noted above. However, the secreted proteins are much more reproducible and more easily characterised than DOC/NOM and as such are easier to standardise, and are anyway secreted into the test system over the exposure time-course, such that utilising them as the NM dispersant will lead to enhanced reproducibility.

Currently the OECD protocols claim to not incorporate any biomolecules though this is not strictly true. Even in a completely biomolecule-free medium, the moment *D. magna* are added to the medium, they themselves release biomolecules so that the medium actually contains biomolecules not currently being accounted for, which impacts the stability of the NMs that are being exposed and therefore toxicity results are being reported incorrectly. We propose that dispersing NMs in conditioned medium, rather than in NOM, presents more controlled and repeatable results as NOM is difficult to characterize and also has seasonal and regional variations. Given that *D. magna* as well as other organisms condition their medium and that this conditioning is reproducible under laboratory conditions and is occurring regardless throughout the 24–48 h acute exposure, the suggestion is to explicitly account for this by using pre-conditioned medium to disperse the NMs. Conditioning of the medium is an easy step towards modifying these protocols so that they are more accurate and reproducible.

The OECD guidance document alludes to the fact that protocols can be developed to ensure alternative testing strategies are appropriate for NMs under realistic conditions and gives an example of how NMs could be incubated in environmental waters (OECD No. 80), although there are no specific and direct rules to follow allowing adaptation to be at the hands of the experimentalist, making inter-study comparison different and hampering uniformity throughout the field. Thus, direct

comparison and ranking of NMs toxicity would be better reported if NMs were incubated in medium previously conditioned by the specific test organism so that the reported toxicity is reflective of real environmental conditions.

Biomolecules, either secreted from *D. magna* itself or present within fresh waters in the form of NOM, create an 'eco-corona', which affects the shape, stability and ultimately the identity of the NMs, which can then further interact with receptors on cellular surfaces instigating internal response. Indeed, NMs in serum-free medium are more toxic towards cells that those exposed with a serum-corona, for example, as the bare NMs pull out biomolecules from the cell membrane in order to reduce their free energy and then enter cells through holes created from the damage caused by the biomolecule removal from the membrane (Lesniak et al., 2012) though this is only one mode of action. In the absence of realistic environmental conditions, which have an abundance of natural biomolecules, the extrinsic toxicity of NMs will differ from the intrinsic toxicity providing an unrealistic EC_{50} as a result of non-passivated surfaces. Biomolecules present in the medium can also influence the NM stability, either stabilizing or destabilizing NM dispersions, leading to enhanced retention in the water column or agglomeration and potential sedimentation. This can be specifically seen for example where 15 nm Au NMs incubated in protein containing medium (specific to a cell study) caused an increase in their aggregation index due to the proteins causing instability of the NMs over 24 h in (Albanese et al., 2014) (within the OECD 202 test time limits) and was also seen with charged PS NMs in protein containing medium from *D. magna* (Nasser and Lynch, 2015). Agglomerated NMs are then a more attractively sized food source for ingestion by *D. magna* making them increasingly toxic as *D. magna* are filter feeders and consume particulates from the water column.

D. magna is able to selectively take up particulates based on size and texture and have been shown to preferentially take up material closer to the size of their natural food source between 1 and 2 μm (Ebert, 2005). There are several instances where the presence of natural biomolecules within a NM suspension mitigates toxicity; for example, the EC_{50} values for CuO NMs and their released ions were two orders of magnitude higher (meaning toxicity was two orders of magnitude lower since the lower the Effective Concentration the more toxic something is) when measured in natural river water containing natural organic components compared to artificial fresh water containing only salts, showing a correlation between the presence of organic matter and EC_{50} values, though interestingly, no such correlation was found for ZnO NMs (Docter et al., 2015) where dissolution is likely the driving factor for toxicity. It was also shown that for ZnO NMs, that where the dissolution occurs (externally in the medium in which case the exposure is to Zn^{2+} ions, or internally within organisms following uptake of the ZnO NMs) also matters in terms of the observed toxicity (Briffa et al., 2018). Biomolecules can also promote or delay the dissolution process, depending on whether there is a strong affinity for the metal ions (Ostermeyer et al., 2013), and whether this affinity promotes retention of ions by the NM or complexes it reducing bioavailability, or drives dissolution and increases the free ion concentration and bioavailability. For example, *D. magna* secreted biomolecules were found to enhance the release of Pb^{2+} ions from alkyl-halide perovskite NMs thus increasing the toxicity arising from exposure to the NMs (Nasser et al., unpublished data). The current OECD standard protocols ignore the myriad roles of biomolecules and their ubiquity in the environment, thereby reducing the relevance of the tests and leading to false results regarding the toxicity of NMs.

The adaptation of current OECD protocols to include characterization of how the presence of biomolecules affects the stability of the NMs in the chosen media would be a first step to understanding the influence of biomolecules on the stability and toxicity of NMs. Another step would be to assess how different concentrations of biomolecules affect stability of NMs over time, which would indicate if NMs continue to stabilize or destabilize depending on how long they are incubated within biomolecule contained medium. Size measurements should be taken at the time of dispersion, as well as during exposure to correctly track the nature of the NMs at different time points in order to accurately report toxicity and risk. Identification of the biomolecules bound to the NMs would be a further step forward, potentially providing additional insights into modes of uptake and toxicity, and facilitating grouping and read-across approaches to be applied to predict ecotoxicity of NMs.

5. Conclusions

In conclusion, it is clear that a food source and natural organic constituents such as those present in environmental waters both need to be included into OECD 202 short term immobilization and long term OECD 211 reproduction tests as both parameters have a large influence on NM uptake, retention within the gut and ultimately NM toxicity towards *D. magna* and their reported risk. Addition of food is a minor change and doesn't require detailed investigation as the same algae used for culturing can be utilised. The simplest way to add natural organic constituents would be to add natural organic matter (NOM), although it is clear that this can introduce additional variability due to the complexity of NOM, although there are standards available. An alternative that might be easier to control and quantify, would be to use conditioned medium, whereby the test organisms are allowed to condition the media for a period prior to addition of the NMs thereby providing a background of biomolecules that can bind to the NMs forming the eco-corona. We have recently shown for *D. magna* that the biomolecules secreted by neonates and by juveniles 3–5 days old are largely similar, although with a few additional proteins secreted by the older organisms. Thus, age-matched and species-matched conditioning and a standard conditioning protocol could easily be worked out such that the protein concentrations reflected those of real environments (2–20 mg/L), and this approach would also be applicable to other test species ensuring consistency across test guidelines.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ssci.2019.05.045.

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